Developmental Biology **240,** 247–253 (2001)
doi:10.1006/dbio.2001.0440, available online at http://www.idealibrary.com on IDEAL®

Persistent Expression of *Hlxb9* in the Pancreatic Epithelium Impairs Pancreatic Development

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The homebox gene *Hlxb9*, encoding Hb9, exhibits a dual expression profile during pancreatic development. The early expression in the dorsal and ventral pancreatic epithelium is transient and spans from embryonic day (e) 8 to e9–e10, whereas the later expression is confined to differentiating β-cells as they appear. We previously showed that *Hlxb9* is critically required for the initiation of the dorsal, but not the ventral, pancreatic program. Here, we demonstrate the requirement for a stringent temporal regulation of *Hlxb9* expression during early stages of pancreatic development. In transgenic mice, where *Hlxb9* expression, under control of the *Ipf1/Pdx1* promoter, was extended beyond e9–e10, the development of the pancreas was drastically perturbed. Morphological analyses showed that the growth and morphogenesis of the pancreatic epithelium was impaired. Moreover, differentiation of pancreatic endocrine and exocrine cells was diminished and instead the pancreatic epithelium with its adjacent mesenchyme adopted an intestinal-like differentiation program. Together, these data point to a need for a tight temporal regulation of *Hlxb9* expression. Thus, a total loss of *Hlxb9* expression results in a block of the initiation of the dorsal pancreatic program, while a temporally extended expression of *Hlxb9* results in a complete impairment of pancreatic development.

Key Words: **homeobox gene; *Hlxb9*; *Ipf1*; *Pdx1*; temporal regulation; pancreatic agenesis; intestinal differentiation.**

INTRODUCTION

The development of the pancreas begins with the dorsal and ventral protrusion of a region of the primitive gut epithelium, and all pancreatic cell types subsequently derive from these endodermal cells of the upper, duodenal, region of the foregut (Edlund, 1998; Fontaine and Le Douarin, 1977; Pictet *et al.*, 1976). Although our knowledge regarding the identity and function of factors involved in pancreatic cell differentiation is increasing, the molecular steps that prior to the onset of the homeobox gene *Ipf1/Pdx1* act to specify early stages in the program of differentiation of the dorsal and ventral pancreatic buds remain unclear. Studies on the role of the homeobox gene *Hlxb9*, encoding Hb9 (Harrison *et al.*, 1994; Ross *et al.*, 1998), during pancreatic development have added interesting insights regarding both early events of pancreatic specification and differences in early development of the dorsal and ventral pancreases (Harrison *et al.*, 1999; Li *et al.*, 1999). *Hlxb9* is transiently expressed in regions of endoderm that give rise to the respiratory and digestive tubes as well as the dorsal and ventral pancreatic anlage (Harrison *et al.*, 1999; Li *et al.*, 1999). In the embryonic pancreases, Hb9 is strongly expressed at e8, starts to disappear already at e9.5, and is virtually gone by e10 (Li *et al.*, 1999). Hb9 expression reappears in the pancreas at later embryonic stages but is then restricted to the insulin-producing β-cells (Harrison *et al.*, 1999; Li *et al.*, 1999). Dorsal pancreatic development is blocked in mice lacking *Hlxb9* function. In contrast, the ventral pancreas develops and contains both endocrine and exocrine cells but the relative proportions and spatial organisation of the various endocrine cells in the ventral pancreas are perturbed (Harrison *et al.*, 1999; Li *et al.*, 1999).

The selective ablation of the dorsal pancreas is striking in view of the early, widespread pattern of *Hlxb9* expression throughout the primitive dorsal endoderm (Li *et al.*, 1999). Both Hb9 and IPF1/PDX1 are expressed transiently at the early stages of pancreatic development when the pancreatic buds form. The early temporal expression of Hb9 in the early pancreatic anlagen appear however more restricted as the high level of Hb9 expression observed at e8 is markedly reduced already 1 day later, whereas IPF1/PDX1 is expressed at high levels until e10 (Figs. 1A–1G; Li *et al.*, 1999).

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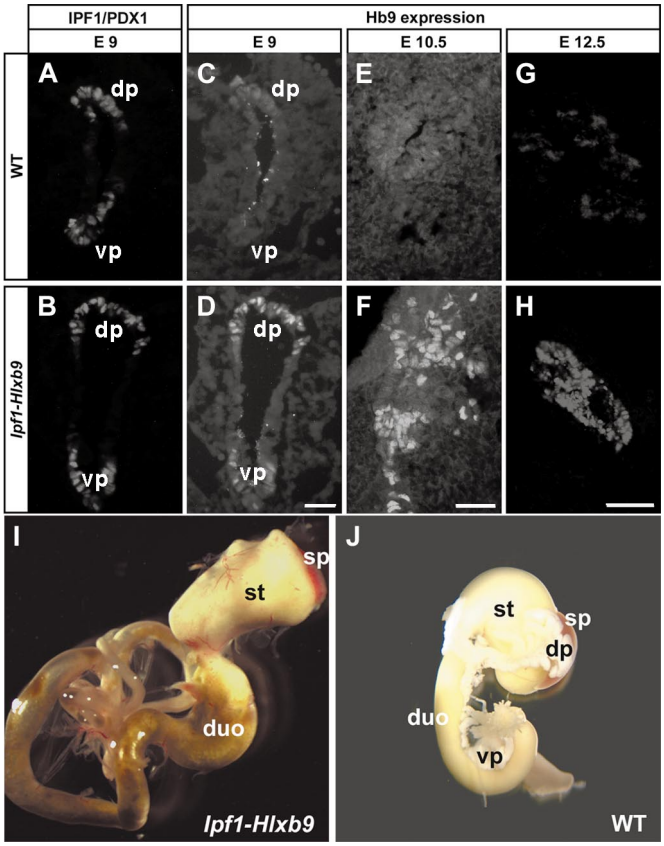

FIG. 1. Persistent *Hlxb9* expression leads to pancreatic agenesis. (A, B) Dorsal and ventral pancreatic IPF1/PDX1 expression in *Ipf1/Hlxb9* transgenic e9.5 embryos (B) was indistinguishable from that of wild-type littermates (A). (C–H) Hb9 expression was enhanced at e9–e10 and maintained beyond e9–e10 in *Ipf1/Hlxb9* transgenic embryos (D, F, H) as compared with the transient expression of Hb9 in wild-type littermates (C, E, G). (I, J) *Ipf1/Hlxb9* transgenic pups survived the fetal development but died soon after birth. Gross examination of the gastrointestinal tract showed that *Ipf1/Hlxb9* transgenic pups (I) completely lacked a pancreas and that the duodenum and stomach appeared enlarged and malformed (I), as compared with wild-type littermates (J). Abbreviations: st, stomach; sp, spleen; duo, duodenum; dp, dorsal pancreas; vp, ventral pancreas. Scale bars in (A–D), 0.02 mm; (E, F), 0.02 mm; (G, H), 0.05 mm.

The expression of Hb9 appears to precede that of IPF1/PDX1 in the dorsal pancreatic anlage, while Hb9 expression in the ventral anlage appears concurrent with that of IPF1/PDX1 (Li *et al.*, 1999). The expression of both proteins reappears in later differentiated β-cells. Nevertheless, comparison of the phenotype of *Hlxb9*- and *Ipf1/Pdx1*-deficient mice reveals important differences in the function of these two genes. Most significantly, *Hlxb9* appears to control an earlier step in the specification of the dorsal pancreatic program, while *Ipf1/Pdx1* acts at a subsequent step in pancreatic development in both dorsal and ventral regions of the pancreas. It still remains an open question whether the dependence of dorsal pancreatic differentiation on *Hlxb9* reflects a function intrinsic to the gut epithelium or whether it reflects a role for *Hlxb9* in the notochord. *Hlxb9* is transiently expressed by notochord cells between ~e8 and e10, and the notochord has been suggested to be instrumental for the initiation of pancreatic development (Hebrok *et al.*, 1998; Kim and Melton, 1997). Thus, it is possible that *Hlxb9* acts to control dorsal pancreatic specification by regulating the expression of inductive or repressive factors secreted from the notochord. To further explore the function of *Hlxb9* during pancreatic development, we used a gain-of-function approach involving a temporal extension of *Hlxb9* expression beyond e9–e10 *in vivo* in transgenic mice. Here, we present data from the analyses of these transgenic mice providing evidence for the need of a tight temporal regulation of *Hlxb9* expression.

METHODS

Preparation of Construct for Transgenic Mice

A 4.5-kb *Not*I–*Nae*I fragment located immediately upstream of the *Ipf1/Pdx1* gene (Apelqvist *et al.*, 1997) was subcloned into a vector carrying a poly(A) site and a 2.2-kb *Eco*RI–*Eco*RI fragment of full-length rat *Hlxb9* cDNA. Transgenic mice were generated by pronuclear injection of the purified expression cassettes (*Afl*III-*Bam*HI) (2.0 ng/ml) into F_2 B6/CBA hybrid oocytes as described in Hogan *et al.* (1994). The genotypes were determined by PCR analyses of genomic DNA extracted from yolk sac or tail tips. The primers used were: 5′-ACAGTGTTAAGTGACCTAGAA-3′ (*Hlxb9* primer for 5′) and 5′-TCGACCTGCAGGCATGCAAGC-3′ (vector primer for 3′). A total number of 17 transgenic mice (n = 2–4 transgenic mice per litter and stage) were analysed.

In Situ Hybridisation and Immunohistochemistry

In situ hybridisation, immunohistochemical localisation of antigens, double-label and whole-mount immunohistochemistry were carried out as described in Apelqvist *et al.* (1997). DIG-labelled RNA probes of rat *Shh* (Apelqvist *et al.*, 1997), mouse *Ihh* (Bitgoood and McMahon, 1995) (kindly provided by A. P. McMahon), and *ngn3* (Apelqvist *et al.*, 1999) were used. Primary antibodies used were: rabbit anti-IPF1/PDX1 (Ohlsson *et al.*, 1993), rabbit anti-Isl1 (Thor *et al.*, 1991), rabbit anti-HB9 (Arber *et al.*, 1999) (kindly provided by T. M. Jessell), rabbit anti-p48 (raised against a GST-p48 fusion protein by AgriSera AB, Vännäs, Sweden), guinea pig anti-insulin (DAKO), guinea pig anti-glucagon (Linco), rabbit anti-carboxypeptidase A (ANAWA), rabbit anti-human α-amylase (Sigma), rat and Cy3-conjugated mouse anti-α-smooth muscle actin (Sigma). When double staining was carried out, a second blocking step using swine anti-rabbit IgG (DAKO) was included. The secondary antibodies used were: Cy3 anti-rabbit (Jackson), fluorescein anti-guinea pig (Jackson), fluorescein anti-rat (Jackson), biotinylated anti-rabbit (Vector), biotinylated anti-rat (Vector), and biotinylated anti-goat (Vector). Streptavidin-FITC (Jackson) and streptavidin-Cy3 (Jackson) were applied to detect biotinylated secondary antibodies.

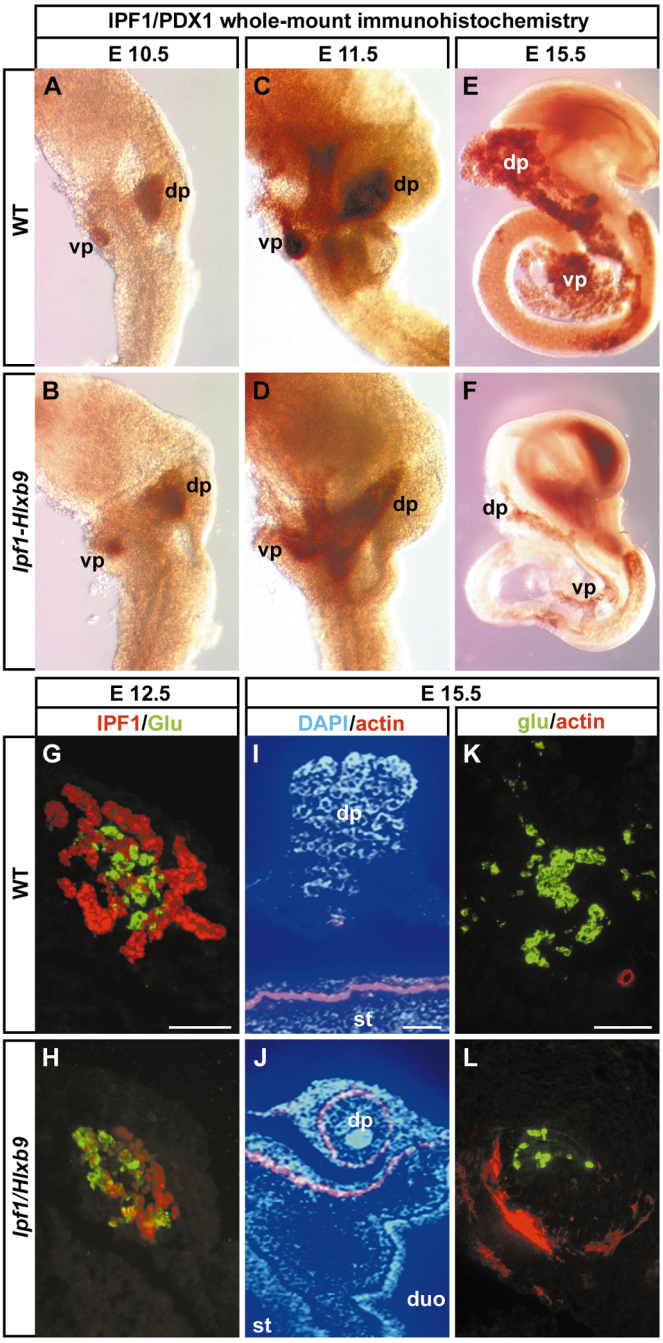

FIG. 2. The growth and morphogenesis of the *Ipf1/Hlxb9* transgenic pancreas is impaired already at early embryonic stages. (A–F) Anti-IPF1/PDX1 whole-mount immunohistochemistry was used to visualise the structure of the pancreatic epithelium at e10.5 (A, B), e11.5 (C, D), and e15.5 (E, F). At e10.5, a relatively normal shaped dorsal and ventral pancreatic bud was detected in the *Ipf1/Hlxb9* transgenic embryos (A, B) but already 1 day later the shape of both the dorsal and ventral pancreatic buds showed an atypical, elongated form (C, D). The perturbed development of the pancreatic epithelium in the transgenic pups became fully evident at e15.5 (E, F). Glucagon-staining (G, H, K, L) was used to locate and confirm the pancreatic region and staining with anti-smooth muscle α-actin antibodies (I–L) showed that the arrested pancreatic epithelium of e15.5 *Ipf1/Hlxb9* transgenics was surrounded by a layer of smooth muscle α-actin$^+$ cells. Note that in wild-type embryos (I, K), smooth muscle α-actin$^+$ staining was only observed lining the vascular epithelium. Abbreviations: st, stomach; duo, duodenum; dp, dorsal pancreas. Scale bars in (G, H), 0.5 mm; (I, J) 0.1 mm; (K, L), 0.1 mm.

RESULTS AND DISCUSSION

Extended Pancreatic Expression of Hlxb9 in Vivo Perturbs Pancreatic Development

To test whether the down-regulation of Hb9 expression at early stages of pancreatic development is important, we extended the expression of *Hlxb9* in the developing pancreatic epithelium of transgenic mice by placing it under the control of the *Ipf1/Pdx1* promoter (Apelqvist *et al.*, 1997). As compared with wild-type littermates, the use of the *Ipf1/Pdx1* promoter prevented the down-regulation of Hb9 expression in the pancreatic buds at stages later than e8 (Li *et al.*, 1999) since a high level of Hb9 expression was still observed at e9, e10.5, and e12.5 in the *Ipf1/Hlxb9* transgenic mice (Figs. 1A–1H). The *Ipf1/Hlxb9* transgenic mice survived the fetal development and were born alive but died shortly after birth. Gross examination of the gastrointestinal tract revealed a complete lack of pancreas or pancreatic tissue, a smaller spleen, a largely dilated duodenum, and an irregular shaped, bulging stomach as compared with control litter mates (Figs. 1I and 1J). These observations suggest that the part of the primitive gut epithelium that normally should have developed into the dorsal and ventral pancreas instead have become part of the developing stomach and duodenum, hence the distended appearance of these two structures.

To try to define the stage at which the development of the *Ipf1/Hlxb9* transgenic pancreas became perturbed, we performed whole-mount immunohistochemical analyses using anti-IPF1/PDX1 antibodies. At e10.5, the pancreatic anlagen of *Ipf1/Hlxb9* embryos expressed apparently normal levels of IPF1/PDX1 and did not look significantly different from that of wild types (Figs. 2A and 2B). By e11.5, the dorsal and ventral pancreatic protrusions of the transgenic embryos display a different, more elongated, tube-shaped form compared with the distinct round-shaped pancreatic structures of stage-matched wild-type embryos (Figs. 2C and 2D). The perturbed development of the *Ipf1/Hlxb9* transgenic pancreases was further advanced in e15.5 transgenic embryos, and, at this stage, two underdeveloped, poorly branched, elongated protrusions were found alongside the developing stomach (i.e., the dorsal protrusion) and duodenum (i.e., the ventral protrusion) (Figs. 2E and 2F). Analyses of sectioned e12.5 dorsal pancreas using anti-IPF1/PDX1 and anti-glucacon antibodies further demonstrated the impaired growth and morphogenesis of the pancreatic epithelium in *Ipf1/Hlxb9* transgenic mice in comparison with control littermates (Figs. 2G and 2H). TUNEL assays

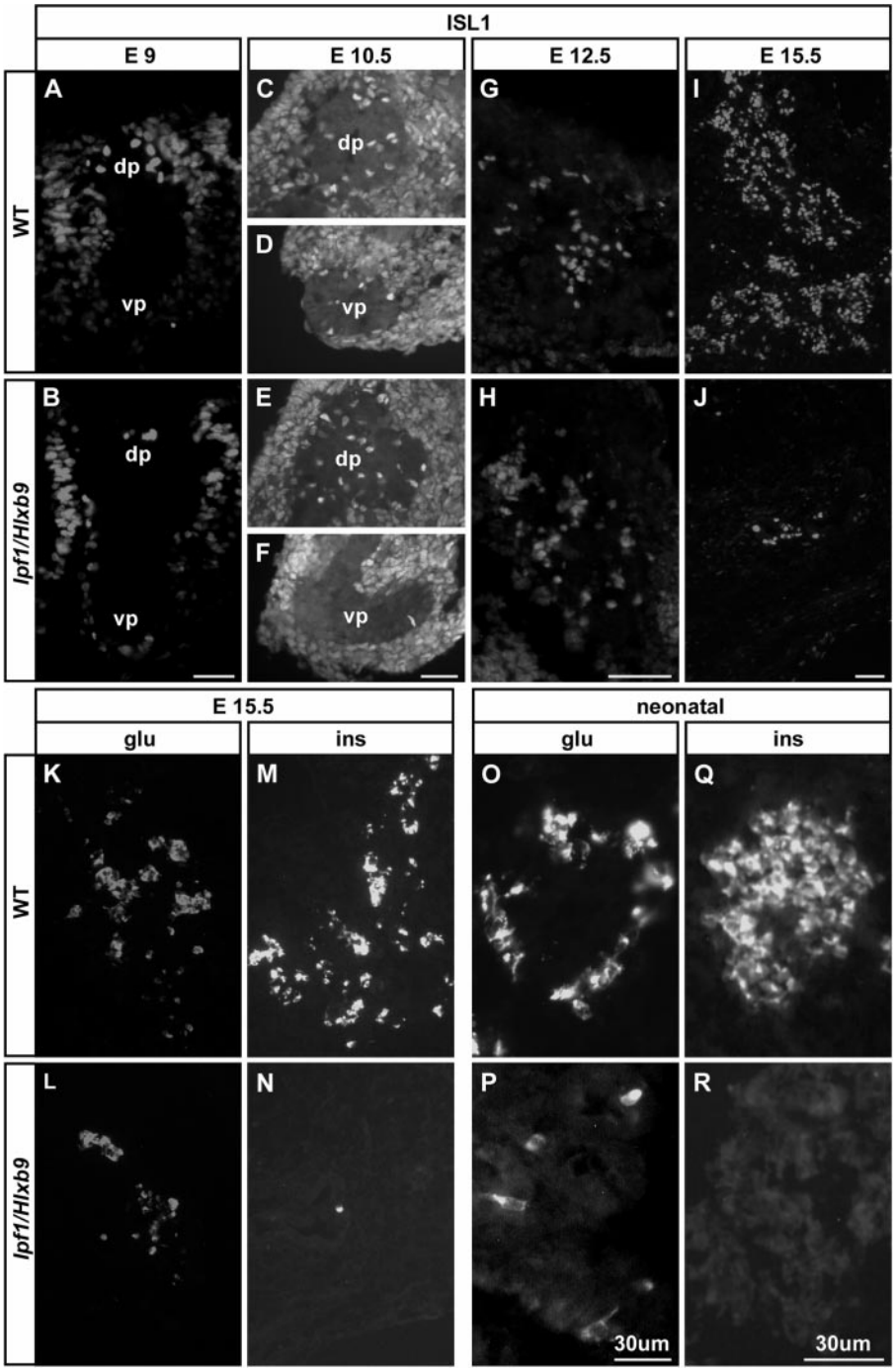

FIG. 3. Initial, but not later stage, pancreatic endocrine cell differentiation was normal in the *Ipf1/Hlxb9* transgenic mice. (A–J) Endocrine cells visualised by ISL1 expression were detected from e9 through e15.5 in both wild-type (A, C, D, G, I) and transgenic pancreatic epithelia (B, E, F, H, J). The expansion of pancreatic endocrine cells between e12.5 and e15.5, however, did not occur in the *Ipf1/Hlxb9* transgenic embryos (G–J). In addition, the endocrine cells present in the transgenic pancreas were predominantly glucagon$^+$ cells (K, L, O, P) and only occasional insulin$^+$ cells could be detected (M, N, Q, R). Abbreviations: dp, dorsal pancreas; vp, ventral pancreas. Scale bars in (A, B), 0.02 mm; (C–F), 0.02 mm; (G, H), 0.05 mm; (I–N), 0.05 mm; (O, P), 0.03 mm; (Q, R), 0.03 mm.

of transgenic and wild-type embryonic pancreases failed to reveal an increased apoptosis in the pancreatic epithelium or mesenchyme of transgenic mice, providing evidence that the observed hypoplasia of the pancreas in *Ipf1/Hlxb9* transgenic mice is not the result of increased programmed cell death (data not shown). Together, these data show that

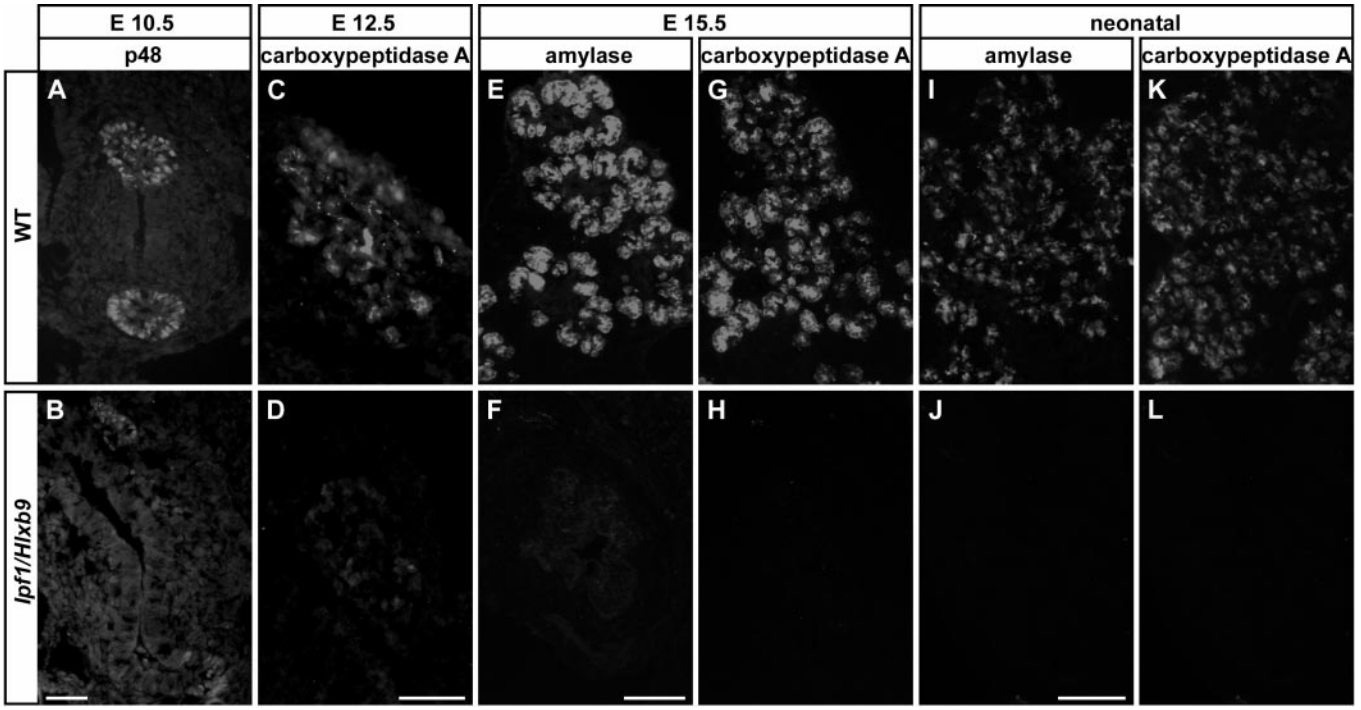

FIG. 4. Pancreatic exocrine cell differentiation is perturbed in *Ipf1/Hlxb9* transgenic mice. (A, B) At e10.5, the earliest exocrine marker p48, was highly expressed in the dorsal and ventral pancreatic buds of wild-type embryos (A) but only a very faint p48 expression could be detected in pancreatic buds of stage-matched transgenic embryos (B). By e12.5, Carboxypeptidase A-expressing cells have appeared in wild-type pancreas (C), whereas Carboxypeptidase A^+ cells were barely detectable in the transgenic embryos (D). The impaired exocrine cell differentiation was evident by e15.5 (E–H) and neonatal (I–L) stages as shown by the lack of exocrine cell types expressing Carboxypeptidase A (G, H, K, L) or amylase (E, F, I, J) in the transgenic mice (F, H, J, L). Scale bars in (A, B), 0.05 mm; (C, D), 0.05 mm; (E–H), 0.1 mm; (I–L), 0.1 mm.

the pancreatic program is initiated in the *Ipf1/Hlxb9* transgenic mice but that the further growth and morphogenesis of the pancreatic epithelium becomes gradually impaired.

Extended Expression of Hb9 in the Developing Pancreatic Epithelium Promotes an Intestinal-Like Differentiation of the Pancreatic Buds

The lack of pancreatic tissue and overall intestinal-like appearance of the presumptive pancreatic regions of neonatal *Ipf1/Hlxb9* transgenic mice resembled the phenotype of transgenic mice expressing *Sonic Hedgehog* (*Shh*) under the control of the *Ipf1/Pdx1* promoter (Apelqvist *et al.*, 1997). In *Ipf1/Shh* transgenic mice, the ectopic expression of *Shh* in the developing pancreatic epithelium instructs the adjacent mesenchyme to differentiate into smooth muscle cells, resulting in the intestinal-like appearance of the pancreatic rudiments of those mice (Apelqvist *et al.*, 1997). To elucidate whether the persistent expression of Hb9 in the developing pancreatic epithelium resulted in a conversion of the pancreatic mesenchyme into smooth muscle cells, we next analysed the expression of smooth-muscle α-actin in transgenic e15.5 dorsal pancreatic rudiments. A layer of smooth-muscle α-$actin^+$ cells, similar to that surrounding the epithelium of duodenum and the stomach, was found to enclose the impaired e15.5 dorsal pancreatic bud (Figs. 2I–2L). $Glucagon^+$ cells were, however, still detected in the developmentally arrested and smooth-muscle layer circled bud. These findings suggest that, in addition to the impaired growth and branching of the pancreatic epithelium, the pancreatic mesenchyme differentiates into intestinal smooth muscle as a result of the extended, high-level expression of Hb9 in the pancreatic epithelium. These results are suggestive of a both cell-autonomous as well as non-cell-autonomous function for Hb9.

Impaired Differentiation of Pancreatic Cell Types in Ipf1/Hlxb9 Transgenic Mice

To investigate the effect of extended pancreatic expression of Hb9 on the appearance of differentiated traits, we next analysed different stages of pancreatic development in transgenic mice with respect to differentiated markers. IPF1/PDX1 expression (Figs. 1A and 1B) was normal at both dorsal and ventral pancreatic levels of transgenic e9.5 embryos and the appearance of early endocrine cells, as visualised by the expression of ISL1 in the dorsal and ventral pancreatic epithelium, also appeared normal both at

e9.5 (Figs. 3A and 3B) and e10.5 (Figs. 3C–3F). By e12.5, i.e., when the impaired growth of the pancreatic epithelium in transgenics was apparent, differentiated endocrine cells still appeared in apparently normal numbers (Figs. 2G, 2H, 3G, and 3H). Between e12.5 and e15.5, the number of pancreatic ISL1$^+$ endocrine cells normally increase dramatically (Pictet and Rutter, 1972), but this expansion was perturbed in the *Ipf1/Hlxb9* transgenic mice (Figs. 3I and 3J). The relatively fewer endocrine cells present in e15.5 pancreases consisted predominantly of glucagon$^+$ cells (Figs. 3K and 3L) whereas only occasional insulin$^+$ cells were observed (Figs. 3M and 3N). Consistently, although some glucagon$^+$ cells could be observed also at neonatal stages, no insulin$^+$ cells were detected (Figs. 3O–3R).

The expression of the exocrine transcription factor p48 (Krapp *et al.*, 1996, 1998) is readily observed in the pancreatic buds of e10.5 wild-type embryos (Fig. 4A), and p48$^+$ cells appeared also in the transgenic e10.5 pancreas (Fig. 4B). The level of p48 expression appeared, however, greatly reduced in comparison with that of wild-type embryos (Figs. 4A and 4B). Exocrine cells expressing carboxypeptidase A were present in wild-type pancreas on e12.5 (Fig. 4C) but in the transgenic e12.5 pancreas no, or only a very low level, expression of carboxypeptidase A could be detected (Fig. 4D). Analyses of differentiated pancreatic exocrine markers at later stages failed to reveal any carboxypeptidase A$^+$ or amylase$^+$ cells at either e15.5 or neonatal stages (Figs. 4E–4L). These results show that the persistent, high level expression of Hb9 in the pancreatic epithelium not only impaired growth and morphogenesis of pancreatic progenitor cells, but also their differentiation.

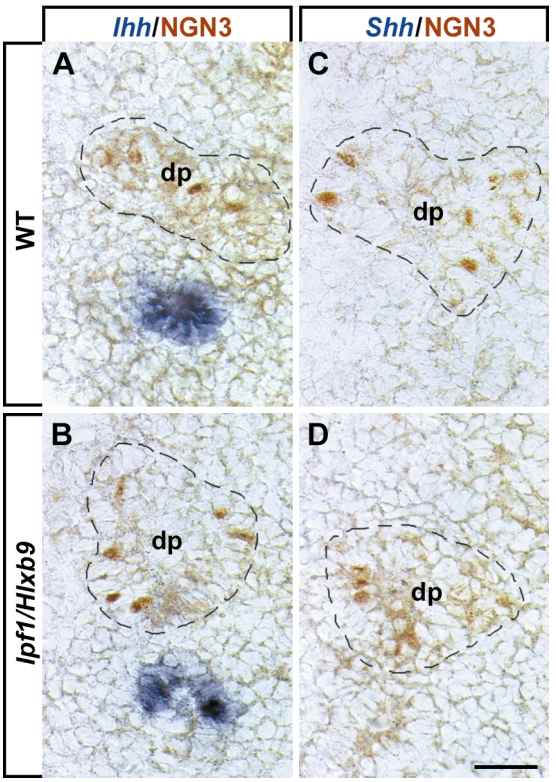

FIG. 5. Normal expression of *hh* in *Ipf1/Hlxb9* transgenic pancreases. (A–D) Analyses of the expressions of *Ihh* (A, B) and *Shh* (C, D) showed that *Ihh* and *Shh* were not ectopically expressed in the pancreatic epithelium of *Ipf1/Hlxb9* transgenic embryos (B, D). *ngn3* was used as a marker to indicate the pancreatic epithelium. Abbreviation: dp, dorsal pancreas. Scale bars in (A–D), 0.02 mm.

Hh Expression Is Still Excluded from the Pancreatic Anlagen in Ipf1/Hlxb9 Transgenics

Both *Shh* and *Indian hedgehog* (*Ihh*) expression commence as two ventrolateral stripes in the gut endoderm on e8.5 and then move dorsally around e9.5 (Echelard *et al.*, 1993; Bitgood and McMahon, 1995), but remain selectively excluded from the pancreatic buds throughout development (Apelqvist *et al.*, 1997). The hedgehog family of proteins has been shown to negatively influence pancreatic development while promoting intestinal differentiation (Apelqvist *et al.*, 1997; Hebrok *et al.*, 1998; Kim and Melton, 1998; Sugekawa *et al.*, 2000). Moreover, the "intestinal phenotype" of the pancreas in *Ipf1/Hlxb9* mice resembled that observed in *Ipf1/Shh* mice (Apelqvist *et al.*, 1997). Hence, we next set out to investigate whether an altered expression of hh molecules could explain the phenotype observed in the *Ipf1/Hlxb9* transgenic mice. No ectopic expression of *Ihh* (Figs. 5A and 5B) or *Shh* (Figs. 5C and 5D) could be observed within the pancreatic epithelium of the *Ipf1/Hlxb9* embryos. Thus, the perturbed and intestinal-like development of the pancreas in *Ipf1/Hlxb9* transgenic mice does not seem to result from ectopic Hedgehog signalling.

The morphogenesis of the pancreatic mesenchyme has been shown to be independent of the concomitant development of the pancreatic epithelium (Ahlgren *et al.*, 1996; Li *et al.*, 1999). The differentiation of the mesenchyme surrounding the stomach and the intestine into intestinal smooth muscle cells is, however, known to be dependent on secretory factors emanating from the adjacent epithelium (Kedinger *et al.*, 1990). In the *Ipf1/Hlxb9* transgenic mice, part of the mesenchyme surrounding the pancreas adopts an intestinal smooth muscle fate. As a consequence, the spleen, which forms from part of the dorsal pancreatic mesenchyme, was smaller in the *Ipf1/Hlxb9* mice than in control littermates (Figs. 1I and 1J, and data not shown). Although *hh* expression was still excluded from the pancreatic epithelium of *Ipf1/Hlxb9* mice, the smooth muscle-like fate of the pancreatic mesenchyme in these mice suggests that maintained expression of Hb9 within the pancreatic epithelium results in non-cell-autonomous effects on the adjacent mesenchyme. These results imply that Hb9 regulates the expression of secretory factor(s) although the nature of these putative factors remains to be identified. Nevertheless, these findings keep open the possibility that Hb9, in addition to a potential cell-autonomous role in the dorsal pancreatic endodermal cells, may control dorsal pancreatic specification and/or induction by regulating the

expression of inductive or repressive factors secreted from the notochord.

ACKNOWLEDGMENTS

We thank K. Falk and U. B. Backman for skillful technical assistance, U. Ahlgren and A. Apelqvist for help with pictures, and members from our laboratory for helpful discussions. This work was supported by grants from the Swedish Society for Medical Research (to H.L.), the Swedish Medical Research Council, the European Commission, and the Juvenile Diabetes Foundation, New York (to H.E).

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Received for publication July 12, 2001
Revised August 23, 2001
Accepted August 24, 2001
Published online October 25, 2001